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Semaphorin3B modulates radiosensitivity of human glioma U-87MG cells

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Abstract This study was to determine the Semaphorin3B (SEMA3B) role in glioma cells responding to irradiation. Two glioma cell lines, which were used here was wild-type p53 (U-87MG), and the other was harboring mutated p53 (U-251). The SEMA3B mRNA could be detected in the two cell lines. The expression level of SEMA3B mRNA was higher in U-87MG cells than in U-251 cells, and increased with time in U-87MG cells after irradiation. Knockdown of SEMA3B expression by shRNA decreased the radiosensitivity of U-87MG cells, this may be associated with the increased G2 accumulation after irradiation. In addition, G2 accumulation after irradiation was enhanced in SEMA3B low-expressing U-87MG cells. These results showed that the SEMA3B was implicated in glioma cells responding to irradiation.

Key words SEMA3B, RNA interference, Ionizing radiation, Radiosensitivity, Glioma

1 Introduction

Semaphorin3B (SEMA3B) as one member of the semaphorin family locates at chromosome region 3p21.3 in human, and encodes a secreted protein belonging to the class 3 semaphorins[1]. Besides axon guidance, the semaphorin3 proteins are implicated in organogenesis[2], neuronal apoptosis[3], immune modulation[4,5], and drug resistance[6,7]. The SEMA3B is frequently inactivated by allele loss and promoter methylation at a relatively early stage of tumor progression[8–12], and considered as a tumor suppressor. The SEMA3B function is mediated by two types of cell surface receptors (neuropilins and plexins)[13–15]. Neuropilins provide binding sites for SEMA3B, and plexins are responsible for its signal transduction[15,16]. The conditioned medium of recombinant SEMA3B -expressing cells can reduce cell growth and induce apoptosis in several lung and breast cancer cell lines [17,18]. As an ovarian cancer cell line, HEY cells expressing SEMA3B exhibit a diminished tumorigenicity in BALB/C$^{nu/nu}$ mice[19], indicating the SEMA3B role in tumor suppression. Also, it is reported in tumor progression[20]. Surprisingly, SEMA3B is expressed at a high level in many invasive and metastatic human tumors. The experimental tumor models confirmed that the SEMA3B expression in tumor cells promotes metastatic dissemination, although this may delay tumor growth[21]. To our knowledge, the SEMA3B role in radiotherapy has not been studied yet.

Glioma as one of the most common malignancies in central nervous system accounts for about 50% of brain tumors, and the high potential invasion is one of its characteristics, impossibly making the complete surgical resection. Radiotherapy is another glioma treatment modality. Because of its radioresistance of glioma, all patients suffer tumor recurrence despite aggressive irradiation. The median survival for glioma patients is approximately one

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year[22,23]. A research based on affimetrix gene chip analysis reported that the SEMA3B expression was associated with poorer overall survival in glioma patients[24]. To date, the SEMA3B role in human gliomas is ambiguous.

In this study, we investigated the correlation between the SEMA3B and the radiosensitivity in glioma cells harboring wild-type p53 (U-87MG) and mutated p53 (U-251) cells. Further, the possible mechanisms were discussed.

2 Materials and Methods

2.1 Cell culture

Human malignant glioma cell lines, U-87 MG and U-251, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were incubated in the 5% CO_2 at 37°C.

2.2 Real-time reverse transcription-PCR (RT-PCR)

Total RNA was extracted from cells with Trizol (Invitrogen, USA) according to the manufacturer's instruction. Reverse transcription (RT) was performed using the Random Primer, dNTP, and PrimeScript Reverse Transcriptase (TaKaRa, Japan). Quantitative real-time PCR was performed in triplicate using SYBR Green Master mix (ABI, USA) on ABI Prism 7000 System. The PCR conditions were 50°C for 2 min; and 95°C for 10 min; and 42 cycles at 95°C for 15 s; and 60°C for 1 min. SEMA3B as 18 s primers included these sense, 5′-AGACTTTCAGCCTGGAG CGAAC-3′ and antisense, 5′-GCAAATGGGT GCGGTTGTAG-3′; sense, 5′-ACGACCCATTCGAA CGTCTG-3′ and antisense, 5′-CCGTTTCTCAGGCT CCCTC-3′. The 18 s was used as an endogenous control. The relative SEMA3B mRNA levels were calculated as follows: ΔCt (sample) = Ct (SEMA3B) − Ct (18 s); $\Delta\Delta CT$=ΔCt (radiation dose point or post-irradiation time point)—CT (0 Gy or 0 h), and relative expression = $2^{(-\Delta\Delta Ct)}$.

2.3 Construction and transfection of short hairpin RNA (shRNA) plasmid expression vector

19-mer hairpin sequences were designed, and the sequences of small interfering RNA constructs targeting SEMA3B (GenBank ID: NM_001005914.1) were as follows: sense, GCUCAGUAUAAUCCCA AAUUG and antisense, CAAUUUGGGAUUAUA CUGAGC. A scrambled sequence of siRNA with no homology of human sequences was used as a control. The oligonucleotides were synthesized, annealed, and ligated into the linearized pGPU6/Neo siRNA Expression Vector (GenePharma, China). To generate SEMA3B shRNA transfected U-87MG cells, the U-87MG cells were seeded into 6-well plates at 3×10^5 per well. When the cells grow to an 80% confluence, the plasmid DNA (4 μg) was transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, the cells were placed into the selection medium containing 200 μg/mL G418 (Amresco, USA) for two weeks. The stably transfected cells, which were named as U-87MG/SEMA3BshRNA or U-87MG/ controlshRNA, were cultured in medium containing the 100 μg/mL G418.

2.4 Western blot analysis for expression of SEMA3B protein

The cells were washed by phosphate-buffered saline (PBS), and lysed in lysis buffer for 40 min. After centrifuging, the supernatants containing proteins were collected. The proteins were electrophoresed on a 5–10% gradient SDS-PAGE gel, and transferred to PVDF membranes. After blocking with 5% nonfat dry milk in PBS containing 0.2% Tween-20, the membrane was probed with anti-SEMA3B antibody (Lifespan, USA) at 1:1000, and followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime, China). Immunoblot signals were detected using ECL reagent (Beyotime). As a control of sample loading, the β-actin expression level was determined using mouse anti-β-actin and HRP-labeled goat anti-mouse IgG (Beyotime).

2.5 Cell irradiation

The irradiation equipment (Soochow University) was used for all radiation-related experiments. Growing exponentially cells were irradiated by the ^{60}Co gamma -radiation source at the dose rate of 0.6 Gy/min.

2.6 Clonogenic survival assay

Cells irradiated with different doses were trypsinized. The cultured cells were appropriately diluted, and replaced in 6-well plates for colony formation assay. The cells were incubated in routine medium at 37ºC for 10 days. All the cells were fixed with 10% methanol, and stained with Giemsa. Colonies of 50 cells or more were counted. The plating efficiency (*PE*) and survival fraction (*SF*) were calculated as follows. *PE* = (number of colonies counted)/(number of un-irradiated cells seeded)×100%, and *SF* = (number of colonies counted)/(number of cells seeded×*PE*). The normalized survival fraction relative to unirradiated cells was calculated. Survival curves were obtained and analyzed by SigmaPlot to get D_0, D_q, and SF_2.

2.7 Cell cycle analysis by flow cytometry

For cell cycle analysis, exponentially growing cells were synchronously cultured by serum deprivation, irradiated with a dose of 4 Gy, and incubated for 24 h at 37°C. Then cells were harvested, washed, and fixed with 70% ice-cold ethanol overnight. The cells were incubated in propidium iodide (50 mg/mL in PBS, containing 100 mg/mL RNase A) at 4°C for 1 h, and analyzed by flow cytometry.

2.8 Statistical analysis

Data were expressed as mean ±standard deviation (±*SD*), and analyzed by SAS statistical software. Student's t-test was used to measure statistical significance between two groups. Multiple comparisons were performed with a one-way analysis of variance (ANOVA). The *p*-value of less than 0.05 was considered as statistically significant.

3 Results

3.1 Expression of SEMA3B mRNA in U-87MG and U-251 cell lines

To study the SEMA3B role in glioma cells exposed to irradiation, the SEMA3B expression in U-87MG and U-251 cells was compared with real-time PCR. The SEMA3B mRNA could be detected in the two glioma cells. The SEMA3B mRNA level was higher in U-87MG cells than in U-251 cells (p<0.05) (Fig.1).

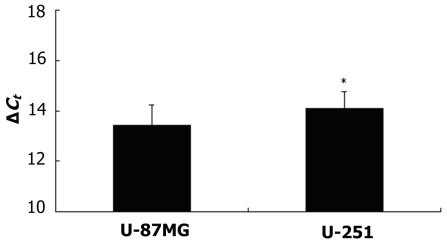

Fig.1 SEMA3B mRNA expression in U-87MG and U-251 cells. The SEMA3B mRNA between U- 87MG and U-251 was compared with real-time RT-PCR. Data are means ±*SD*, *n*=3. The asterisk represents statistical significance compared to U-87MG cells (p<0.05).

3.2 Effect of irradiation on SEMA3B mRNA expression in glioma cell lines

In U-87MG, the SEMA3B mRNA level had an irradiation-induced increase (Fig.2A), its highest level was in cells irradiated by 4 Gy, and about 2.41-fold higher than the unirradiated U-87MG cells.

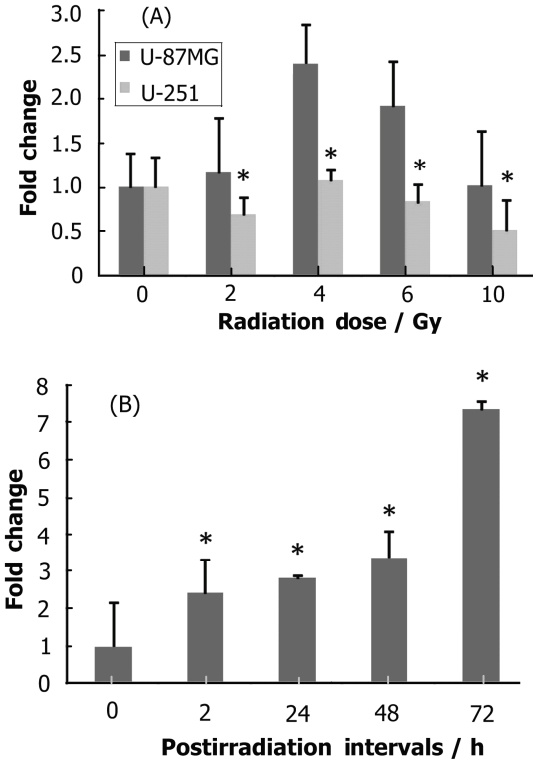

Fig.2 Effect of irradiation on SEMA3B production in glioma cell lines (A) Normalized SEMA3B mRNA expression in U-87MG and U-251 cells irradiated by different doses. Results represented means ±*SD* (*n*=3). The asterisk represented statistical significance compared to unirradiated cells (p<0.01). (B) SEMA3B mRNA levels in U-87MG at different time point after irradiation. Data here are means ±*SD* (*n*=3). The asterisk represents statistical significance compared to cells 0 h after irradiation (p< 0.01).

However, the SEMA3B mRNA expression in U-251 cells had a radiation-induced decrease, its lowest level were in the two cells treated with 10 Gy. Comparing the U-87MG with the U-251, a statistically significant increase in SEMA3B expression was observed for each dose point (p<0.05). As shown in Fig.2B, following a single irradiation dose of 4 Gy, the SEMA3B mRNA level in U-87MG cells had a time dependent increase. Maximal increase was at 72 h after post-irradiation.

3.3 Knockdown of SEMA3B by shRNA transfection

The real-time RT-PCR and Western blots were performed to assess the effect of SEMA3B shRNA on SEMA3B expression. The inhibition rate of SEMA3B mRNA after stable transfection with SEMA3B shRNA was 97% (Fig.3A). Compared with U-87MG/control shRNA, the expression of SEMA3B protein was down regulated significantly in U-87MG/SEMA3BshRNA cells by Western blots (Fig.3B). These results show that the cells were stably transfected with inhibited expression of SEMA3B.

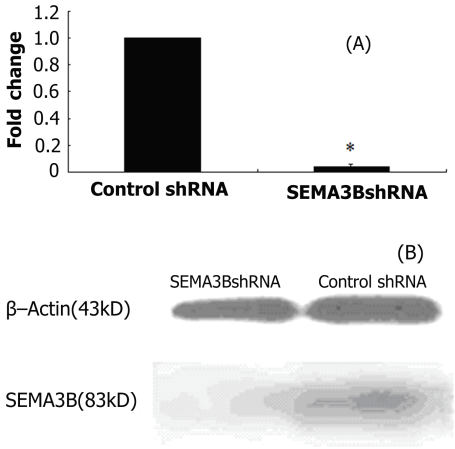

Fig.3 Inhibitory effect of SEMA3B shRNA on SEMA3B expression. (A) The expression of SEMA3B mRNA in U-87MG/SEMA3BshRNA and U-87MG/controlshRNA cells. Data are presented as means ±*SD* (n=3). The asterisk represented statistical significance compared with U-87MG /controlshRNA cells (p<0.01). (B) Comparison of SEMA3B protein between U-87MG/SEMA3BshRNA and U-87MG/ controlshRNA by Western blotting.

3.4 Knockdown of SEMA3B decreased radiosensitivity of U-87MG cells

As radiation enhanced SEMA3B mRNA expression in U-87MG cells, the down regulated SEMA3B in the radiosensitivity of U-87MG cells was examined. The radiosensitivity of cells was evaluated by performing the clonogenic survival assay. Compared with control cells, our data demonstrated that the survival fraction of U-87MG/SEMA3BshRNA cells significantly increased for each dose point (p<0.05). The survival curves show that the down regulation of SEMA3B enhanced the colony forming ability of U-87MG cells (Fig.4). The values of D_0, D_q, and SF_2 were calculated by Sigma Plot (Fig.4B), indicating that SEMA3B decreased the radiosensitivity of U-87MG cells.

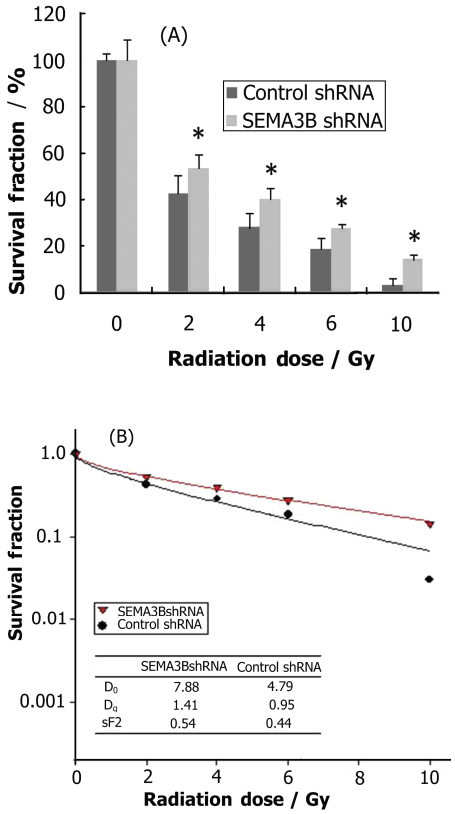

Fig.4 Knockdown of SEMA3B expression decreased the radiosensitivity of U-87MG cells. (A) Cells irradiated with 0, 2, 4, 6 and 10 Gy were assayed for cell survival using clonogenic assay. Data are presented as means ±*SD* (n=3). Asterisks represented statistical significance compared with U-87MG/ controlshRNA (p<0.05). (B) The survival curves were fitted by SigmaPlot software according to the linear-quadratic model.

3.5 Knockdown of SEMA3B expression enhanced G2 accumulation of U-87MG after irradiation

To explore the mechanism that SEMA3B modulates the radiosensitivity of U-87MG cells, the cell cycle distribution exposed to 0 or 4 Gy was analyzed. After irradiation, the G2 phase percentage in U-87MG/ SEMA3BshRNA and U-87MG/controlsh RNA cells

increased, which was more evident in U-87MG/ SEMA3B shRNA than in U-87MG/ controlshRNA cells (Fig.5). Moreover, there was the G1 accumulation in U-87MG/controlshRNA but not U-87MG/SEMA3B shRNA cells. This may be the mechanism of SEMA3BshRNA-induced radio-resistance in U-87MG.

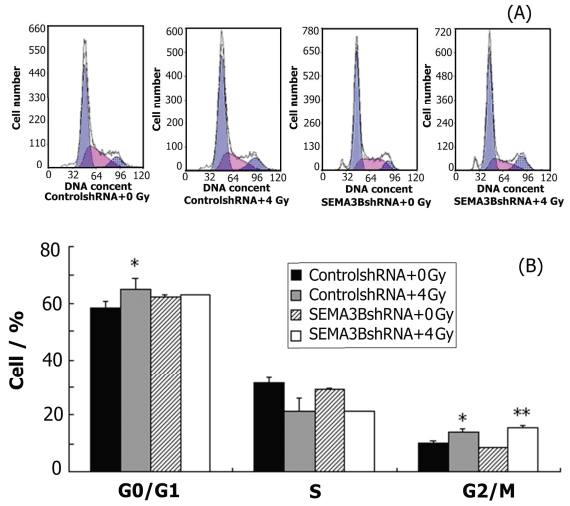

Fig.5 Cell cycle analysis of U-87MG/SEMA3BshRNA and U-87MG/controlshRNA cells by flow cytometry. (A) Representative figures of cell cycle distribution. (B) The cell cycle distribution of cells exposed to 0 or 4 Gy and harvested at 24 h post-irradiation. Data were means ±*SD* (n=3), *statistical significance compared with U-87MG/controlshRNA irradiated with 0 Gy. **statistical significance compared with U-87MG/ SEMA3BshRNA irradiated with 0 Gy (p<0.05).

4 Discussion

The SEMA3B role in irradiated glioma cell lines was investigated. The SEMA3B mRNA expression was higher in U-87MG cells than in U-251 cells. The irradiation enhanced SEMA3B expression of U-87MG in a time-dependent manner. The SEMA3B mRNA was the highest level when cells were irradiated by 4 Gy, but the irradiation induced SEMA3B mRNA decreased in U-251 cells. The SEMA3B differently responded to radiation due to the genetic nature of glioma cell lines. This difference between different glioma cells may be ascribed to the p53 status. As we know, the U-87MG is a glioma cell line with wild-type p53, and U-251 harbored the mutated p53. The SEMA3B in axon guidance is a direct target of p53 and inducible by p53. The introduction of exogenous p53 into a glioma cell line lacking wild- type p53 can dramatically induce the SEMA3B mRNA expression, and the endogenous SEMA3B expression was induced in response to UV irradiation in p53-dependent manner[25], this result was consistent with that cells were exposed to gamma irradiation.

Further, the radiation-enhanced SEMA3B expression in U-87MG was examined, the expression of SEMA3B by RNA interference was down regulated, thus acquiring the stably transfected U-87MG cells because the SEMA3B expression was suppressed. The RNA interference is a technique in which the double stranded RNA (dsRNA) was used to target specific mRNAs for degradation, thereby silencing the specific gene expression[26]. Although the dsRNA, which is able to silence gene expression, was only discovered a few years ago, it has become an important tool to analyze gene function extended into a broad diversity of organisms, including human cells[27]. By this method, the SEMA3B may be correlated with the radiosensitivity of U-87MG cells. Because the U-87MG cells transfected with SEMA3B shRNA was more resistant to irradiation than control cells. The cell cycle distribution shows that knockdown of SEMA3B expression in U-87MG improved G2 accumulation after irradiation. The cellular radiosensitivity is determined by a number of fundamental processes. Cell cycle distribution is one of the factors affecting cell radiosensitivity[28]. Cell cycle delay at G2 is considered as a process that the DNA damage was repaired[29]. So, the increased G2 cells and the promoted more cells repairing DNA damage may be the mechanism of SEMA3B modulating the radiosensitivity of U-87MG cell.

In addition, the SEMA3B could inhibit the phosphatidylinositol 3-kinase (PI3K)/Akt pathway through neuropilin-1 in lung and breast cancer cells. Activation of PI3K/AKT pathway is implicated in radioresistance, and associated with the three major radiation resistance mechanisms: intrinsic radio-sensitivity, tumor cell proliferation and hypoxia[30]. The PI3K/AKT pathway may be used another mechanism as SEMA3B modulating cell radio-sensitivity. Also, the SEMA3B competed with vascular endothelial growth factor (VEGF) for binding to the receptor neuropilin[31], and the VEGF signaling is related with the radioresistance. The VEGF inhibition enhanced radiation sensitivity[32–34]. These

results may give the mechanism how SEMA3B modulates cell radiosensitivity.

5 Conclusions

The SEMA3B was implicated in the glioma cells responding to irradiation. The radiation enhanced SEMA3B mRNA expression of U-87MG was in a time dependent manner. Knockdown of SEMA3B expression increased the radioresistance of U-87MG cells, this may be associated with the increased G2 accumulation after irradiation. Our study may help to understand the SEMA3B role in glioma radiotherapy.

References

1 Yazdani U, Terman J R. Genome Biology, 2006, **7:** 211.1–211.14.

2 Behar O, Golden J A, Mashimo H, *et al.* Nature, 1996, **383:** 525–528.

3 Gagliardini V, Fankhauser C. Mol Cell Neurosci, 1999, **14:** 301–316.

4 Hall K T, Boumsell L, Schultze J L, *et al.* Proc Nat Acad Sci USA, 1996, **93:** 11780–11785.

5 Kumanogoh A, Kikutani H. Trends Immunol, 2001, **22:** 670–676.

6 Sekido Y, Bader S, Latif F, *et al.* Proc Nat Acad Sci USA, 1996, **93:** 4120–4125.

7 Yamada T, Endo R, Gotoh M, *et al.* Proc Nat Acad Sci USA, 1997, **94:** 14713–14718.

8 Castro-Rivera E, Ran S, Thorpe P, *et al.* Proc Nat Acad Sci USA, 2004, **101:** 11432–11437.

9 Sekido Y, Bader S, Latif F, *et al.* Proc Nat Acad Sci USA, 1996, **93:** 4120–4125.

10 Tomizawa Y, Sekido Y, Kondo M, *et al.* Proc Nat Acad Sci USA, 2001, **98:** 13954–13959.

11 Tse C, Xiang R H, Bracht T, *et al.* Cancer Res, 2002, **62:** 542–546.

12 Tischoff I, Markwarth A, Witzigmann H, *et al.* Int J Cancer, 2005, **115:** 684–689.

13 Tamagnone L, Artigiani S, Chen H, *et al.* Cell, **99:** 71–80.

14 Takahashi T, Fournier A, Nakamura F, *et al.* Cell, 1999, **99:** 59–69.

15 Rohm B, Ottemeyer A, Lohrum M, *et al.* Mech Dev, **93:** 95–104.

16 Kolodkin A L, Matthes D J, Goodman C S. Cell, 1993, **75:** 1389–1399.

17 Yoshio T, Yoshitaka S, Masashi K, *et al.* Proc Nat Acad Sci USA, 2001, **98:** 13954–13959.

18 Castro-Rivera E, Ran S, Thorpe P, *et al.* Proc Nat Acad Sci USA 2004, **101:** 11432–11437.

19 Christin T, Xiang R H, Bracht T, *et al.* Cancer Res, 2002, **62:** 542–546.

20 Neufeld G, Lange T, Varshavsky A, *et al.* Adv Exp Med Biol, 2007, **600:** 118–131.

21 Rolny C, Capparuccia L, Casazza A, *et al.* J Exp Med, 2008, **205 :** 1155–1171.

22 Stupp R, Mason W P, van den Bent M J, *et al.* N Engl J Med, 2005, **352:** 987–996.

23 Stupp R, Hegi M E, Mason W P, *et al.* Lancet Oncol, 2009, **10:** 459–66.

24 Jeremy N R, Hans C, Jones B, *et al.* Cancer Res, 2005, **65:** 4051–4058.

25 Kensuke O, Mori T, Toyama Y, *et al.* Neoplasia, 2002, **4:** 82–87.

26 Zamore P D. Science, 2002, **296:** 1265–1269.

27 Dudley N R, Goldstein B. Curr Opin Mol Ther, 2003, **5:** 113–117.

28 Dote H, Burgan W E, Camphausen K, *et al.* Cancer Res, 2006, **66:** 9211–9220.

29 Sancar A, Lindsey-Boltz L A, Unsal-Kacmaz K, *et al.* Annu Rev Biochem, 2004, **73:** 39–85.

30 Schuurbiers O C, Kaanders J H, van der Heijden H F, *et al.* J Thorac Oncol, 2009, **4:** 761–767.

31 Castro-Rivera E, Ran S, Thorpe P, *et al.* Proc Nat Acad Sci USA, 2004, **101:** 11432–11437.

32 Sandström M, Johansson M, Bergström P, *et al.* J Neurooncol, 2008, **88:**1–9.

33 Williams K J, Telfer B A, Shannon A M, *et al.* Br J Radiol, 2008, **1:** S21– S27.

34 Knizetova P, Ehrmann J, Hlobilkova A, *et al.* Cell Cycle, 2008, **7:** 2553–2561.